Saprolegnia bulbosa sp. nov. isolated from an Argentine stream: taxonomy and comparison with related species

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Abstract

Saprolegnia bulbosa sp. nov. was isolated from floating and decaying twigs and leaves in El Gato stream, Partido de La Plata, Buenos Aires Province, Argentina. The distinctive characteristics of *S. bulbosa* are the product of smooth oogonia and predominantly contorted monoclinous, androgynous and diclinous antheridia. The oogonial stalks are usually bent, curved or once coiled; oospores are subcentric, (1) 2–15 (45) per oogonium and are variable in size. Taxonomical description of this new species, its comparison with related oomycetes of the genus and the nucleotide sequences of the internal transcribed region (spacers ITS1, ITS2 and the gene 5.8S) of its rRNA gene are given here.

Introduction

Water molds grow on submerged and floating leaves, twigs and wood in well-aerated waters. These are commonly known as Oomycetes and were historically classified as belonging to the Kingdom Fungi. However, unlike other eumycetes (true fungi), the members of oomycetes remain diploid throughout their life cycles, with meiosis occurring in the gametangia before fertilization (Alexopoulos *et al.*, 1996). Sexual reproduction is by gametangial contact and the male gametes are entirely replaced by male nuclei, which are directly injected into the oospores present in the female gametangia. Ultrastructural, biochemical and molecular analyses of the oomycetes suggest that these are closer to Algae (Phaeophyta) and higher plants (Cavalier-Smith, 1986; Margulis *et al.*, 1989). Presently, the oomycetes are no longer considered to be true fungi and are now classified among 'Stramenopiles', one of the eucaryotic divisions that includes water molds and brown algae (Patterson & Sogin, 1992; Dick, 2001a, b). This position of oomycetes, unrelated to true fungi but closely related to heterokont (brown) algae, has now been well established using molecular phylogenies that are based on rRNA gene sequences (Kumar & Rzhetsky, 1996; Paquin *et al.*, 1997; Van de Peer & De Wachter, 1997).

The genus *Saprolegnia* is very well known and is cosmopolitan in distribution. It mainly consists of aquatic fungi like oomycetes, living mainly as saprophytes but also as fish parasites. *Saprolegnia parasitica* is the best known out of all the known parasites of the genus. The disease caused is generally known as Saprolegniosis of fish and their eggs (Johnson *et al.*, 2002).

During a study of zoosporic organisms occurring in water and floating organic matter in streams and channels of Partido de La Plata, Buenos Aires Province, Argentina, some oomycetes, belonging to the genus *Saprolegnia*, were isolated. Three of these resembled each other but were quite different from those described in the literature. The type specimen is now being described as a new species *Saprolegnia bulbosa* Steciow. According to the present classification, it belongs to the Kingdom Straminipila, Phylum *Heterokonta*, Class *Peronosporomycetes* (Alexopoulos *et al.*, 1996; Dick, 2001a, b). Members belonging to the family of *Saprolegniaceae* are cosmopolitan and yet very little known; they are difficult to key out and their identification is mainly based on features of the reproductive structures, that is, the size and shape of oogonia, antheridia and sporangia. Recently, however, the morphological descriptions of a species have been increasingly supplemented by its molecular characteristics (Lévesque *et al.*, 1998). The PCR of the internal transcribed (ITS) region of the rRNA gene and its sequencing has become a useful tool for the species separation, identification and determination of their natural

relationships and for ascertaining genetic diversity. Along with the investigations on morphology, physiology and pathogenicity, the ITS sequence data provide valuable information on 'new' or undescribed taxa. Together with morphological differences, the sequence diversity can support the creation of new species (Paul *et al.*, 1999; Paul, 2001; Paulitz *et al.*, 2003; Paul & Steciow, 2004). Some recent works on the genus *Saprolegnia* have been carried out in Argentina (Steciow, 1998, 2001, 2003; Steciow & Elíades, 2002), but all these works were based on morphological observations. The taxonomy of the genus is mainly based on the morphological descriptions provided by different authors from time to time (Coker, 1923; Sparrow, 1960; Seymour, 1970), but the most comprehensive (and freely available on the internet) work is that of Johnson *et al.* (2002).

Saprolegnia bulbosa sp. nov. from Argentina is being described. Both morphological details of the oomycete and the sequence of the ITS region (spacers ITS1, ITS2, and the gene 5.8S) of its rRNA gene are taken into consideration.

Materials and methods

Oomycetal materials

Zoosporic organisms were isolated by the methods described earlier by Coker (1923), Sparrow (1960), Seymour (1970) and Fuller & Jaworski (1987). Samples of brown decaying twigs and leaves and wood of the local dominant vegetation (*Senecio bonaerensis, Sagittaria montevidensis*, *Ludwigia peploides*, *Salix* spp.) collected from El Gato stream were brought to the laboratory in separate sterile polyethylene bags. These samples were placed in sterilized petridishes containing sterile distilled water and several hemp seed halves (*Cannabis sativa*) and were incubated at room temperature (15–20 °C). Once the seeds were colonized, a single hypha or sporangium was isolated under the microscope and transferred to a cornmeal agar medium (CMA). After 3–4 days, a block of agar from the edge of each colony was cut off and placed in sterilized petridishes containing distilled water and hemp seeds halves in order to obtain new colonies. Development of sporangia was induced by changing water periodically. Measurements and observations were made on those colonies growing in water cultures. Temperature–growth relationship was observed by incubating these at 5°, 10° and 25 °C. The diameters of the oomycete colonies, diameters of oogonia, number of oospores per oogonia and diameters of oospores were calculated from 50 counts of each of three replicates. Measurements and observations were made using an Olympus BX 40 microscope (Olympus Optical Co. Ltd, Tokyo, Japan) equipped with phase-contrast optics. The total percentage of the type of antheridial branches was calculated from all of these replicates. The type specimen is deposited in the mycological herbarium of the Spegazzini Institute (LPS) and its culture collection (LPSC).

DNA isolation and PCR

The oomycetes were grown in PDB (potato dextrose broth), which is prepared in the same manner as PDA (potato dextrose agar) without the addition of agar-agar. The culture conditions, DNA isolation and the PCR amplification of the ITS of the ribosomal nuclear DNA were performed using the procedures described elsewhere (Chen *et al.*, 1992; Paul *et al.*, 1998, 1999, Paul, 2001). Universal primers ITS1 and ITS4 were synthesized and the DNA sequence was realized by Oligo Express (Paris). The ITS1 sequence of *S. bulbosa* was compared with the ITS1 sequences of other related species. The sequence of the ITS region of the nuclear ribosomal DNA of this oomycete has been deposited in Genbank.

Results

Taxonomy

Saprolegnia bulbosa Steciow (Figs 1 and 2)

Mycelium densum, cultura in seminibus Cannabis sativae 1–4 cm diam. Hyphae ramosa, pleraque 10–67 μm late diam. ad basim. Sporangia in culturis juvenilibus, cylindrica, filiformia vel naviculata, (229 –) 255–521 (– 735) μm longa et (20 –) 25–41 (– 50) μm lata, renovata per proliferationem internam. Ejecto sporarum pro genus typica, zoospori incystatis globosi 9–15 μm. Gemmae parcus. Oogonia copiosa, pyriformia vel sphaerica, (35 –) 45–97 (– 102) μm diam. Paries oogonia laevis, ramulus lateralibus provenientia, 10–533 μm. Tumoribus hypharum lateralibus se inter oogonia intermiscentibus. Oospori (1 –) 2–15 (– 45) per oogonium, subcentrici, (10 –) 15–35 (– 41) μm diam. Ramulus antheridiales, ramosus, plerumque origine monoclina (41% ± 4) et androgyna (34% ± 2%) sed interdum diclina (25% ± 3).

The mycelium is extensive, denser near the substratum; 2-week-old hemp seed colony is 1–4 cm in diameter in water culture (reaching 1.5 ± 0.5 cm at 5 °C; 3.0 ± 0.5 cm at 10 °C and 3.5 ± 0.5 cm at 25 °C). Main hyphae are slender, sparingly branched and measure 10–67 μm at the base (near the seeds). Oogonia-like hyphal swellings are present which can be spherical or subglobose, laterally attached, intermingled with true oogonia, but their contents do not divide to form oospheres. Gemmae are scanty, spherical, pyriform, clavate or irregular, simple or catenulate. Zoosporangia are formed plentifully and are mostly cylindrical, filiform or naviculate; (229 –) 255–521 (– 735) × (20 –) 25–41 (– 50) μm; and

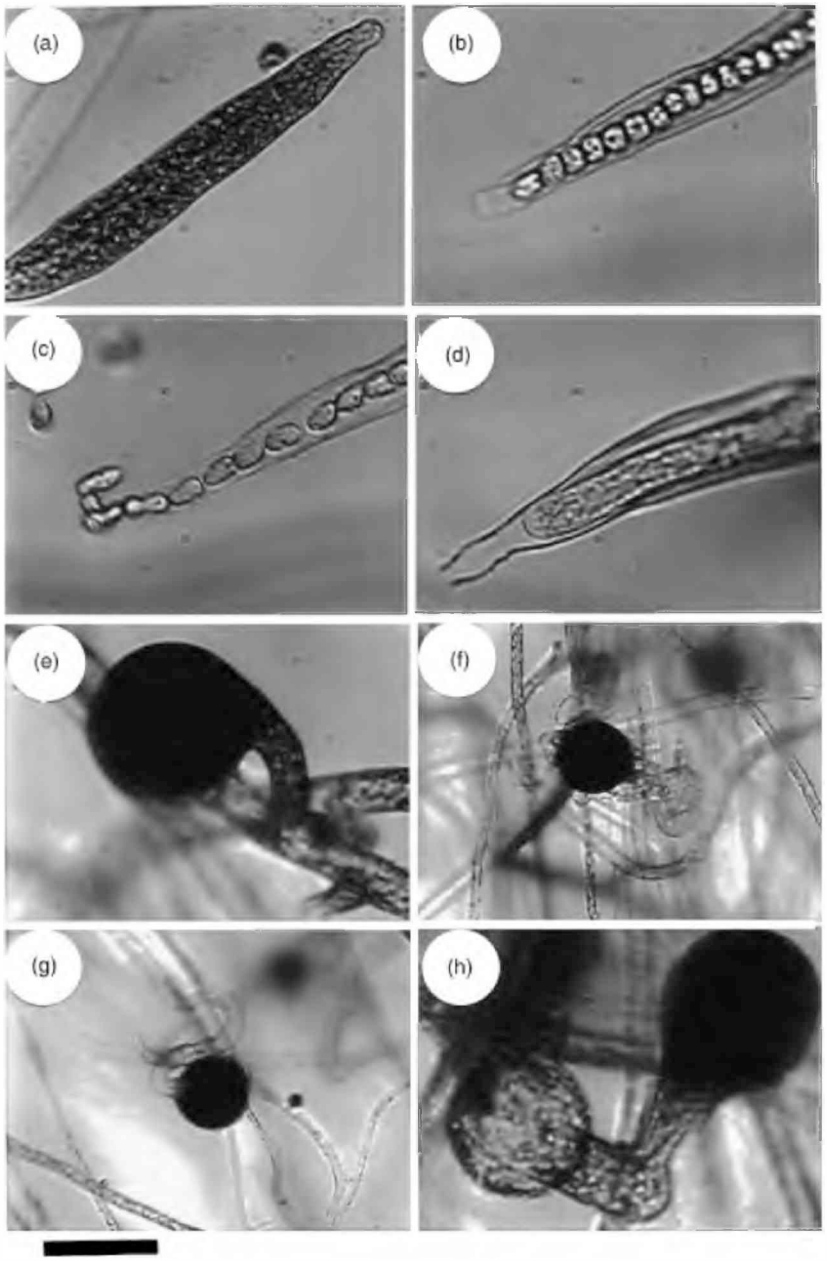

Fig. 1. (a–h) *Saprolegnia bulbosa*. (a) Details of a naviculate zoosporangium containing zoospores. (b–d) Detail of zoospores' discharge; zoosporangium renewal by internal proliferation leads to the development of a new one inside. (e–h) Immature oogonia with curved or once coiled oogonial stalk and some of them with a characteristic bulbous oogonial stalk and antheridial branches. Scale bar: a–e, h = 66.6 μm; f–g = 210 μm.

variable in size, often with one or two discharge papillae, terminal (Fig. 1a–d). The sporangial renewal is usually by internal proliferation (Fig. 1d) and these are formed in basipetalous succession. Zoospore discharge is typically saprolegnoid (Fig. 1b and c). Encysted spores are globose, 9–15 μm diameter. Oogonia are very abundant, terminal or lateral, rarely intercalary; pyriform, spherical or with a lateral projection; (35 –) 45–97 (– 102) μm; immature oogonia frequently proliferating (Fig. 1e–h). Oogonial wall are smooth, pitted or pitted only under the point of attachment of antheridial cell. Oogonial stalks are frequently stout and variable in length, bent, curved or coiled (Fig. 1e, g) with a suboogonial swelling (Fig. 1 h), very rarely slender and straight when short and 10–533 μm long. Antheridial branches are principally monoclinous (Fig. 2a–c, g–h), androgynous and bulbous (Fig. 2e), occasionally diclinous (Fig. 2d, f), branched and often contorted wrapping around the oogonia (Fig. 2c, h). Antheridial cells are simple or branched, attached by projections or laterally appressed to the oogonia. Oospheres maturing and are subcentric, type I, filling the oogonium (Fig. 2i–l), spherical or ellipsoid; (1 –) 2–15 (– 45) in number; and are (10 –) 15–35 (– 41) μm in diameter.

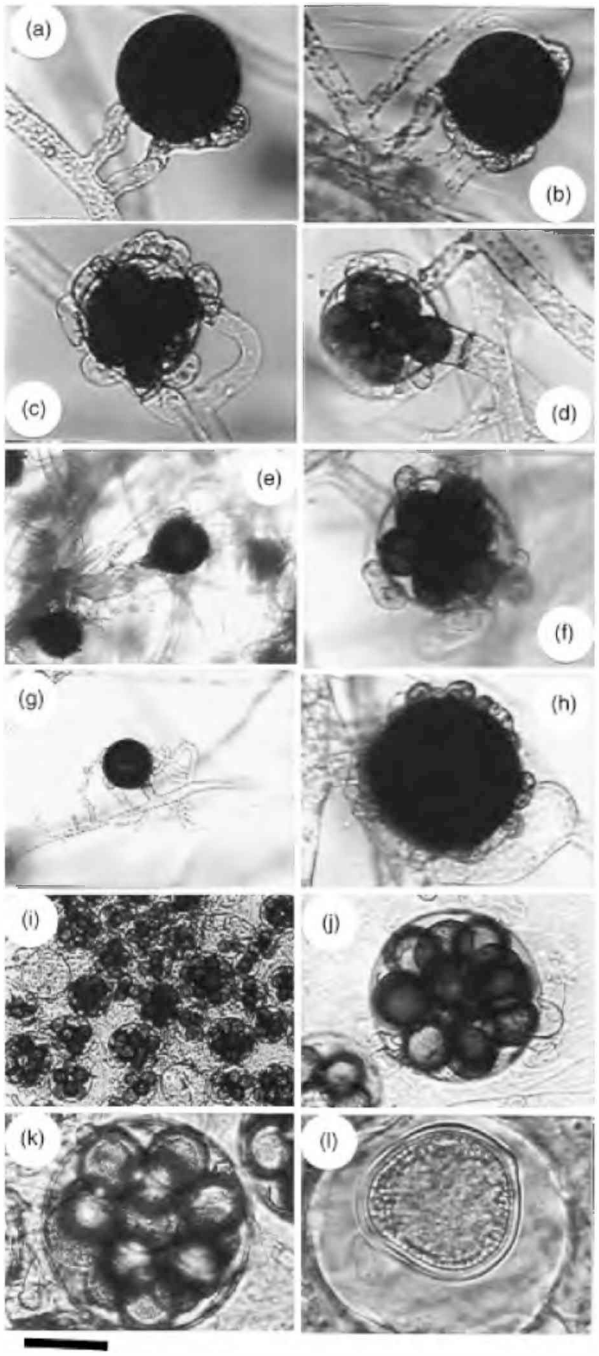

Fig. 2. (a–l) *Saprolegnia bulbosa*. (a, b, g) Characteristic monoclinous antheridial branches on immature oogonia. (c–d) Diclinous antheridial branches on mature oogonia. (e) Androgynous antheridial branch arising from a bulbous oogonial stalk. (f, h) Oogonia with smooth oogonial wall and antheridial cell laterally appressed. (i) Aspect of mycelium with numerous mature oogonia in water culture. (j–k) Smooth oogonia with subcentric oospores inside and pitted oogonial wall. (l) Detail of subcentric oospore, type I. Scale bar: a–d, f, h, j = 66.6 μm; E, G, I = 210 μm; K = 50 μm; L = 20 μm.

Holotype: Argentina. Buenos Aires, La Plata, El Gato stream, on floating litter; June 282001, leg. M.M. Steciow, LPS N° 45800; culture collection LPSC N° 745.

Etymology: The name *S. bulbosa* refers to the characteristic bulbous oogonial stalks, the numerous hyphal swellings occurring in the mycelium and the frequent bulbous androgynous antheridial branches.

The ITS region (and a part of the flanking regions) of the rRNA gene of *S. bulbosa* is comprised of 723 bases. 1–19 = 18S gene, partial sequence; 20–182 = ITS1 complete sequence; 183–329 = 5.8 S gene complete sequence; 330–673 ITS2 complete sequence; 674–723 = 28S gene partial sequence. The sequence has been submitted to the Genbank (Accession AY267 011).

A BLAST search yielded the nearest identity (96.8%) with a new species isolated in France: *Saprolegnia multispora* (Paul & Steciow, 2004); other identities were with *Achlya oligacantha* (80.8%), and *Saprolegnia ferax* (79.9%).

Discussion

The new oomycete, *S. bulbosa*, is characterized by the presence of bulbous oogonial stalks, hyphal swellings in the mycelium and bulbous androgynous antheridial branches. The oospores of the oomycete are subcentric type 1. Antheridial branches and the measurements of oogonia are constant features of this species. There was little variation in the type and size of zoosporangia at different temperatures; they are cylindrical, filiform or naviculate. The shape of the oogonia too remained constant, mainly pyriform or spherical (smooth, rarely with one lateral projection).

Oogonial production was unaffected by the different temperatures; all colonies developed oogonia, but mature oospores were less abundant at lower temperatures.

Saprolegnia bulbosa Steciow appears to be related to *Saprolegnia terrestris* Seymour (1970) in having smooth-walled oogonia, androgynous and monoclinous antheridial branches. However, *S. terrestris* has predominantly androgynous ones, arising from immediately below the oogonia that develop on mainly straight oogonial stalk in the Australian type collection (Seymour, 1970; Khulbe, 2001) and can be bent or coiled in New Zealand strain (Elliott, 1968), but is never bulbous as in the case of this Argentina species. Moreover, *S. bulbosa* has only subcentric oospores as compared with the subcentric (type I or II) and centric oospores of *S. terrestris*, which are smaller, (9 −) 20–25 (− 30) μm in New-Zealand type isolate (Elliott, 1968) and (20 −) 24–32 (− 41) μm in Australian-type collection (Seymour, 1970).

The oogonia of *S. bulbosa*, are at times, provided with lateral projection, and are pitted; the oogonial stalk is often bent or coiled with the characteristic bulbous base, whereas in *S. terrestris*, the oogonia is unpitted or very rarely pitted,

Table 1. Comparison of the morphological features of *Saprolegnia bulbosa* and related species

	S. bulbosa	*S. oliviae*	*S. longicaulis*	*S. variabilis*	*S. multispora*	*S. terrestris*
Zoosporangial shape	Cylindrical, filiform or naviculate	Fusiform, naviculate, (clavate, filiform)	Cylindrical, filiform, Clavate, irregular	Filiform, fusiform, Clavate, irregular	Fusiform, filiform, Clavate, naviculate	Fusiform, pyriform, obpyriform, clavate, spherical; irregular or contorted
Zoosporangial size	(229–) 255–521 (–735) × (20–) 25–41 (–50)	194–600 × 24–50	190–500 (–700) × 20–50	(127–) 137–407 (–485) × 20–49	(97–) 121–485 × 19–50	60–400 × 16–48
Oogonial wall	Smooth, pitted	Smooth (with a papilla or lateral projections or apical outgrowth), pitted	Smooth, pitted	Smooth, pitted	Smooth, pitted	Smooth or sparsely papillae, pitted or unpitted
Antheridial branches	Monoclinous, androgynous and bulbous (diclinous)	Absent (present). Diclinous, androgynous (monoclinous)	Diclinous, (monoclinous)	Diclinous, (monoclinous or androgynous)	Diclinous, (monoclinous or androgynous)	Androgynous, (monoclinous)
Oogonial diam (μm)	(35–) 45–97 (–102)	(29–) 60–140 (–160)	(30–) 60–80 (–115)	(39–) 51–92 (–153)	(29–) 60–111 (–136)	(35–) 60–65 (–91)
Oospore type	Subcentric, type I	Subcentric, type I	Subcentric, type I	Subcentric, type I	Subcentric, type I	Subcentric, type I (centric)
Oospore diam (μm)	(10–) 15–35 (–41)	(12–) 19–30	(12–) 24–30 (–34)	(9–) 15–30 (–56)	(10–) 12–20 (–25)	(20–) 24–32 (–41)
Oopores per oogonium	(1–) 2–15 (–45)	(1–) 15–50 (–70)	(1–) 2–9 (–26)	(1–) 3–18 (–60)	(1–) 11–70 (–100)	(1–) 2–11 (–18)

and are smaller, (35–) 60–65 (–91) μm diameter, having fewer oospores (1–) 3–6 (–12) (Seymour, 1970). The number of oospores can reach up to 30 in New Zealand isolates (Elliott, 1968).

Saprolegnia bulbosa develops numerous bulbous hyphal swellings, which then function as a zoosporangium at the end of the hypha. This is not found in the case of *S. terrestris* and the zoosporangia are shorter, reaching 60–400 μm in length in the Australian-type isolate (Seymour, 1970) or only up to 112 μm in the case of the New Zealand isolates (Elliott, 1968).

Saprolegnia variabilis Steciow & Elíades and *S. multispora* Paul & Steciow also possess subcentric oospores type I developing within smooth, and pitted internal-walled oogonia like those of *S. bulbosa* (Steciow & Elíades, 2002; Paul & Steciow, 2004). However, the oogonial dimensions, number of oospores per oogonia, the nature of antheridial branches and the morphology of zoosporangia are quite different when compared with the new species. Both of these species do not have the bulbous antheridial base and bulbous oogonial stalks as found in *S. bulbosa*.

Saprolegnia bulbosa is also related to *Saprolegnia australis* Elliott and *Saprolegnia longicaulis* Steciow, with subcentric oospores; however, these differ from *S. bulbosa* in having predominantly diclinous antheridial branches, which are never contorted (Elliott, 1968). *Saprolegnia longicaulis* is not known to possess androgynous antheridial branches and the oogonial stalk is much longer (102–1300 μm) than in the case of this new Argentine species (Steciow, 2001).

There is a serious lack of ITS sequences of the members of the genus *Saprolegnia* in the Genbank. A comparative study, hence, becomes very difficult. However, the ITS sequence of this new species brings it very close to some recently described new species like *Saprolegnia oliviae* (98.8%), and *S. longicaulis* (98.8%), *Saprolegnia variabilis* (98.7%). It is interesting to note that between the species, variations in the sequences are as small as just two to three bases. This phenomenon is similar to that found in the case of another oomycete, *Pythium*, in which the different isolates within a species may vary by only one or two base pairs (Paulitz *et al.*, 2003).

The differences in the morphological characteristics between closely related species as compared in Table 1, supplemented by the differences in the ITS region of the rRNA gene of *S. bulbosa*, justify the creation of this new taxon.

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